Exhibit B

Activated protein C versus protein C in severe sepsis

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Objective: To delineate critical differences between activated protein C (APC) and its precursor, protein C, with regard to plasma levels in health and in severe sepsis, and to discuss the implications of these differences as they relate to treatment strategies in patients with severe sepsis.

Data Source/Study Selection: Published literature including abstracts, manuscripts, and review articles reporting studies in both experimental animal models and humans that provide an understanding of the relationship and the critical differences between circulating levels of APC and protein C.

Data Extraction and Synthesis: The protein C pathway represents one of the major regulatory systems of hemostasis, exhibiting antithrombotic, profibrinolytic and anti-inflammatory properties. This pathway also plays a critical role in the pathophysiology of severe sepsis. Central to this pathway is the vitamin K-dependent serine protease, APC, and its precursor, protein C. The conversion of protein C to APC is dependent on the complex of thrombin and thrombomodulin, an integral endothelial surface receptor. The conversion of protein C to APC is further augmented by another endothelial surface protein, the endothelial protein C receptor. There are limited published data on APC levels in health and disease, probably due to the complexity of the assay methodology for measuring APC and the absence of commercially available diagnostic kits. In animals and humans with normal functioning endothelium, circulating levels of APC (1–3 ng/mL) are positively correlated with protein C (4000–5000 ng/mL) concentration and the amount of thrombin generated. In patients with severe sepsis, there is a generalized endothelial dysfunction, contributing to multiple organ failure with increased morbidity and mortality. Persistently low protein C levels are related to poor prognosis. Key to understanding the treatment strategy with APC or protein C is knowledge of the functional status of the endothelium and, specifically, whether the microvasculature in patients with severe sepsis can support the conversion of protein C to APC. To date, only APC (drotrecogin alfa [activated]) has been shown to reduce mortality in severe sepsis in a large, phase 3, placebo-controlled, double-blind international trial. In contrast, no data, other than open-label case studies, are available for evaluation of the effects of protein C in the treatment of severe sepsis.

Conclusion: The limited data available indicate that lower levels of protein C in sepsis occur in the absence of appreciable conversion to APC. These observations indicate that treatment with APC may be more efficacious than protein C in severe sepsis, where generalized endothelial dysfunction may impair conversion of protein C to APC. Additional research is required to confirm these observations. (Crit Care Med 2001; 29[Suppl.]:S69–S74)

KEY WORDS: protein C; activated protein C; sepsis; endothelial protein C receptor; coagulation

Controversy surrounds the potential therapeutic utility of protein C vs. activated protein C (APC) in patients with sepsis. Unfortunately, publications on the topics of APC and protein C sometimes incorrectly use these two terms interchangeably (1). The incorrect use of the nomenclature increases confusion for those readers who are not aware of the complexities of the protein C pathway in health and disease states. The objective of this article is to review and delineate critical differences between protein C and APC with regard to circulating levels and conversion of the precursor, protein C, to APC in experimental animal models, normal subjects, patients without extensive endothelial injury, and patients with severe sepsis. Extensive *in vivo* and *in vitro* studies indicate that APC, the active serine protease, has antithrombic, profibronlytic and anti-inflammatory activities (2–4). In contrast, the precursor protein C has not been shown to have significant biological activity.

Assay Methodologies for Protein C and Activated Protein C

Commercially produced diagnostic kits are readily available for determination of plasma levels of protein C (Fig. 1). There are three different types of assays. One type uses immunoassay methodology to measure antigenic levels of protein C using plasma prepared from anticoagulated blood samples. The other two assay types determine the functional activity of protein C. In both of these two types of diagnostic kits, protein C has to be converted to APC with snake venom proteases, and the activity of APC is measured by either an activated partial thromboplastin time-based assay or an amidolytic-based assay. Citrated plasma samples are needed for both types of protein C functional activity measurements, which can be performed by automated specialty coagulation instruments available in most major hospital laboratories. Because of the ready availability of commercial diagnostic kits and instruments for determination of plasma protein C levels, there is a considerable amount of published data on the plasma levels of protein C in health and disease (5–9).

In contrast, no commercial diagnostic kit is available for the determination of APC levels in plasma, although several methods for determining levels of APC in

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Protein C Assays:
Commercially available kits; standard equipment; rapid

1. Immunoassay : measures antigenic levels
2. Two different functional assays:
   i) PC ⟶ aPC ⟶ aPTT clotting activity
   ii) PC ⟶ aPC ⟶ amidolytic activity

Activated Protein C Assays:
No commercially available kits; non-standard reagents; slow

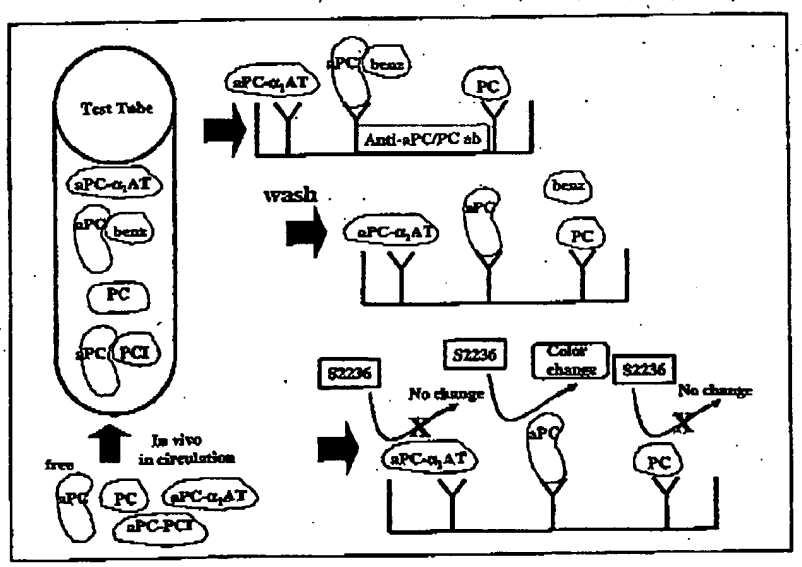


Figure 1. Key differences among assays for both protein C and activated protein C. *PC*, protein C; *aPC*, activated protein C; *aPTT*, activated partial thromboplastin time; α_1AT, α_1-antitrypsin; *benz*, benzamidine; *PCI*, protein C inhibitor.

plasma have been published (10–13). All of these methods involve many steps, requiring from hours to several weeks to perform, and the key reagents are not commercially available.

APC is irreversibly inactivated by several plasma serine protease inhibitors (14–16) and, consequently, has a half-life of about 25 mins, even in a citrated blood sample. For assay methods that quantitate levels of APC directly (Fig. 1) (11, 13), blood samples must be collected with the reversible inhibitor of APC benzamidine in addition to the anticoagulant citrate. Benzamidine blocks the inhibition of activated protein C by the plasma serine protease inhibitors in blood samples. Plasma is prepared as quickly as possible and is stored frozen at −70°C. APC, in the presence of benzamidine, is immuno-captured with a monoclonal antibody that does not block its active site. The excess plasma and the benzamidine are subsequently removed, and the amount of APC is quantitated by its ability to hydrolyze a chromogenic peptide substrate (amidolytic activity), liberating a yellow color.

There are two methods used to quantitate levels of APC indirectly (10, 12). One, developed by Bauer and colleagues (10), quantitates the 12-amino acid peptide released when protein C is converted to APC. The method used by Espana and colleagues (12) requires the collection of two blood samples, one in heparin and the other one in benzamidine and citrate. Heparin accelerates and augments the complete, irreversible, complex formation of APC with plasma serine protease inhibitors, which are subsequently quantitated using immunoassays. The level of circulating APC is marked as the difference in concentration of the APC and serine protease inhibitor complexes between the heparinized blood sample and the citrate-benzamidine blood sample.

Circulating Levels of Protein C and Activated Protein C in Health

Protein C is the zymogen (inactive precursor) of the vitamin K-dependent serine protease APC. In healthy adults, the circulating concentration of protein C is approximately 4000 to 5000 ng/mL (~70,000 pM) (11, 17), whereas the circulating concentration of APC is approximately 1 to 3 ng/mL (~35 pM) (11–13). Thus, protein C is normally found in concentrations approximately 2,000-fold higher than APC. Circulating levels of protein C are lower in normal newborns and children than in adults (18, 19). The circulatory half-life ($t_{1/2\alpha}$) of protein C in humans is about 10 hrs (20–22); in contrast, the circulatory activity half-life ($t_{1/2\alpha}$) of APC in normal subjects, either plasma-derived (7) or recombinantly produced (9), is only about 20 mins. The short half-life of APC activity in circulation is a result of the inhibition of APC by several plasma serine protease inhibitors, such as protein C inhibitor, α_1-antitrypsin, α_2-antiplasmin, and plasminogen activator inhibitor-1 (14–16).

Conversion of Protein C to Activated Protein C in Health and in Conditions Without Generalized Systemic Endothelial Dysfunction

Protein C is converted to APC when thrombin complexes with thrombomodulin, an endothelial surface glycoprotein (23). This conversion is further augmented by another endothelial surface receptor, endothelial protein C receptor (EPCR) (24, 25).

The normal range of circulating levels of protein C has been well established by several large epidemiologic studies (5–7) and varies over approximately a two-fold range between the upper and lower limit of normal (~2,800–5,600 ng/mL or 70% to 140%). In healthy humans, there is a positive correlation between the levels of circulating protein C and APC (12). The

available data appear to support the hypothesis set forth by Esmon (26) that under physiologic conditions in the absence of disease, the conversion of protein C to APC by thrombin-thrombomodulin is dependent on circulating levels of protein C. The Km of protein C, as substrate for thrombin-thrombomodulin, is approximately equal to the normal physiologic plasma concentration of protein C (26).

Thrombin formation modifies the relationship between protein C and APC plasma levels. For example, in healthy baboons, infusion of low concentrations of thrombin generated a proportional and persistent increase in levels of APC (27). Levels of APC increased to 250 to 500 ng/mL from a normal baseline level of 5 ng/mL (representing a 5000% to 10,000% increase) in these baboons, while endogenous levels of protein C decreased by only 15% to 30%. Similarly, in certain prothrombotic states where there is an elevation of thrombin generation without generalized endothelial dysfunction, circulating levels of APC were found to be elevated (10, 28, 29), with less than a two-fold increase from normal baseline. These prothrombotic states include normal aging, genetic predisposition with factor V Leiden (a mutation in factor V that leads to APC resistance), and localized vessel occlusion. The increase in APC levels was found to be positively correlated with markers of thrombin generation, such as prothrombin fragment F1.2, thrombin-antithrombin complex, or fibrinogen fragment A (12, 30–32).

Circulating levels of APC were determined in patients with acute myocardial infarction (17). The range of APC levels in these patients before thrombolytic therapy ranged from 0.15 to 23 ng/mL (mean, 6 ng/mL), with the upper end of the range being approximately ten-fold higher than normal. During streptokinase therapy, levels of APC rose further to a range of 26 to 154 ng/mL (mean, 69 ng/mL). The mechanism of this conversion of protein C to APC during thrombolytic therapy may be a result of the presence of thrombin that is either released from the lysed thrombus or that is generated *de novo* (33). Endogenous protein C levels in this group of patients did not differ from normal and were approximately 5000 ng/mL. It was not unexpected that endogenous protein C levels were minimally affected, even given the 35-fold increase in mean APC levels, because the ratio of protein C to APC was minimally altered. These data suggest that in diseases where there is no generalized systemic endothelial dysfunction, high levels of APC can be generated from endogenous protein C with a minimal reduction of endogenous protein C levels.

Conversion of Protein C to Activated Protein C in Severe Sepsis with Generalized Endothelial Dysfunction

Sepsis is a disease that results from an intense systemic response of the host to an infection. The systemic response includes activation of the inflammatory pathways, activation of the coagulation pathway, impairment of fibrinolytic pathways, and an intricate interplay between the hemostasis and the inflammatory pathways (34, 35). This host response to infection leads to a generalized systemic dysfunction of the endothelium and multiple organ failure (36).

Protein C levels were reported to be below the lower limit of normal in >80% of patients with severe sepsis (37–40). Low protein C levels, which remain depressed in patients with sepsis, are related to poor prognosis (8, 9, 41). Because of the prognostic relationship between low protein C levels in patients with sepsis and increased morbidity and mortality, it was assumed that the decreased levels of protein C in sepsis resulted from increased conversion of protein C to APC. Since APC has a much shorter circulatory half-life compared with protein C, continuous rapid conversion of protein C to APC was assumed to be the principal process leading to the consumption of protein C. Consequently, it was assumed that if low circulating protein C levels could be restored to normal by infusion of exogenous protein C, morbidity and mortality from sepsis might be reduced. This appears to be the basis for the open-label case studies (42–53) and an ongoing small placebo-controlled trial (54) in patients with severe sepsis, where exogenous protein C was used as "replacement" therapy. The key assumption was that the vasculature in patients with severe sepsis could adequately convert protein C to APC, the agent active in reversal of the systemic response to infection and, therefore, in reduction of mortality. Until very recently, there were no data on the levels of APC in experimental animal sepsis models and in patients to support the hypothesis of protein C replacement therapy for severe sepsis. In fact, recent emerging data suggest the opposite: treatment with protein C may not be appropriate for patients with severe sepsis.

A recent study in an experimental baboon sepsis model examined levels of endogenous APC (55). Baboons were administered 10^{11} colony-forming units of *Escherichia coli* intraperitoneally. Following *E. coli* exposure, some of the baboons recovered completely, some sustained illness over a 2-wk period, and some died within 48 hrs. Overall, there was a decrease in endogenous protein C by more than 50%, with a maximum transient increase in endogenous APC of four times normal. Regardless of the outcome, there was a lack of correlation among the decrease in endogenous protein C levels, the elevation of the thrombin generation marker thrombin-antithrombin complex, and the increase in endogenous APC levels. In contrast, healthy baboons infused with low doses of thrombin (27) exhibited endogenous APC levels that persistently increased to between 50- and 100-fold above baseline, with a 15% to 30% concomitant decrease in endogenous protein C levels.

These data are in agreement with a clinical study (37) reporting endogenous APC levels in 26 leukemic patients who developed severe sepsis or septic shock after intense chemotherapy. There was a transient and statistically insignificant increase (median, ~4 ng/mL) in endogenous APC levels in patients who developed septic shock compared with those who developed severe sepsis; patients in septic shock also demonstrated an early decrease in endogenous protein C levels to 40% of normal. Notably, the decrease in protein C levels preceded the transient increase in APC levels. Thus, the small transient rise in APC could not have accounted for the large drop in protein C.

In both the phase 2 and phase 3 trials of severe sepsis with drotrecogin alfa (activated) a recombinant human APC, endogenous APC levels were determined in the placebo group during the first 2 to 4 days of the study. For the phase 2 study (38), the lower limit of detection of the APC assay was 5 ng/mL. The majority of the placebo-treated patients with severe sepsis (32/40; 80%) had no detectable levels of endogenous APC above 5 ng/mL. The remaining placebo-treated patients (8/40; 20%) had APC levels that were detectable (between 5 and 20 ng/mL), but the levels were variable, transient, and exhibited no discernible increasing or decreasing pattern over time. In the double-

blind, placebo-controlled phase 3 trial, recombinant human activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS), endogenous APC was determined in approximately 350 placebo-treated patients over the first four study days. Results similar to the phase 2 study were also observed (39).

In the treatment arm of the phase 3 trial, patients receiving drotrecogin alfa (activated) had significantly lower mortality compared with patients receiving placebo (39). In this trial, patients were dosed with a continuous infusion of drotrecogin alfa (activated) for 96 hrs, resulting in the equivalent of APC levels of approximately 20 times above the normal levels of endogenous APC (39).

Role of Thrombomodulin and Endothelial Protein C Receptor in the Conversion of Protein C to Activated Protein C in Severe Sepsis

In vitro studies have shown that endotoxin and inflammatory cytokines, such as tumor necrosis factor-α can down-regulate endothelial surface thrombomodulin (56–58), either by decreasing synthesis or by increasing degradation. Endothelial surface thrombomodulin may also be cleaved and released into the circulation as soluble thrombomodulin (59). Indeed, the correlation between elevation in circulatory soluble thrombomodulin and diseases with endothelial dysfunction and vascular disorders has been published extensively and reviewed (60). Elevated soluble thrombomodulin has also been reported in animal sepsis models (55) as well as in patients with sepsis (Dhainaut JF, unpublished observations; 61–66). However, the reduction of endothelial surface thrombomodulin at the tissue level *in vivo* in animal sepsis models has not been demonstrated (67, 68).

The other endothelial surface receptor, EPCR, that further augments the conversion of protein C to APC via thrombin-thrombomodulin, was shown by *in vitro* experiments to be up-regulated by endotoxin and thrombin but unaffected by proinflammatory cytokines such as tumor necrosis factor-α. An elevation of circulating soluble EPCR was demonstrated in both an experimental mouse sepsis model (69) and in patients with sepsis (70). In this rodent sepsis model, EPCR was not diminished at the tissue level. These results suggest that elevation of soluble EPCR with maintained tissue levels resulted from a combination of increased shedding and increased endothelial expression of EPCR.

However, preliminary data on endothelial surface levels of thrombomodulin and EPCR in patients with severe sepsis (71) appear to be different from that found in experimental animal sepsis models. Thrombomodulin and EPCR were reduced in skin biopsy samples from 21/21 patients and 17/21 patients, respectively, with meningococcal septicemia. These data suggest that patients with severe sepsis may not have sufficient endothelial surface thrombomodulin and EPCR to convert protein C to APC. Based on this initial observation, Faust and colleagues (72) are currently extending their study to measure endogenous levels of APC in meningococcemic patients, who were treated with protein C. When completed, this study may clarify whether the decrease in endothelial surface thrombomodulin and EPCS in these patients leads to an impairment of the conversion of protein C to APC.

Proposed Mechanism for Impaired Conversion of Protein C to Activated Protein C in Sepsis

The data reviewed regarding the crucial role of endothelial integrity in the process of converting protein C to APC in health and disease are shown in Figure 2. In health, with normal endothelial function (*left panel*), circulating protein C in the vessel lumen is converted to relatively low circulating APC levels by the thrombin-thrombomodulin complex and EPCR. APC levels are dependent on both circulating levels of protein C and thrombin. The *middle panel* represents the disease without generalized endothelial dysfunction, in which protein C is converted to substantially increased levels (35-fold) of APC, without resulting in a significant reduction in protein C levels. The increase in APC may be the body's response to restore hemostatic balance, which is perturbed by disease toward a procoagulant state. The *right panel* demonstrates the events hypothesized to occur during sepsis with generalized systemic endothe-

Proposed Mechanism of Endothelial Dysfunction in Sepsis Resulting in Inability to Convert Protein C to activated Protein C

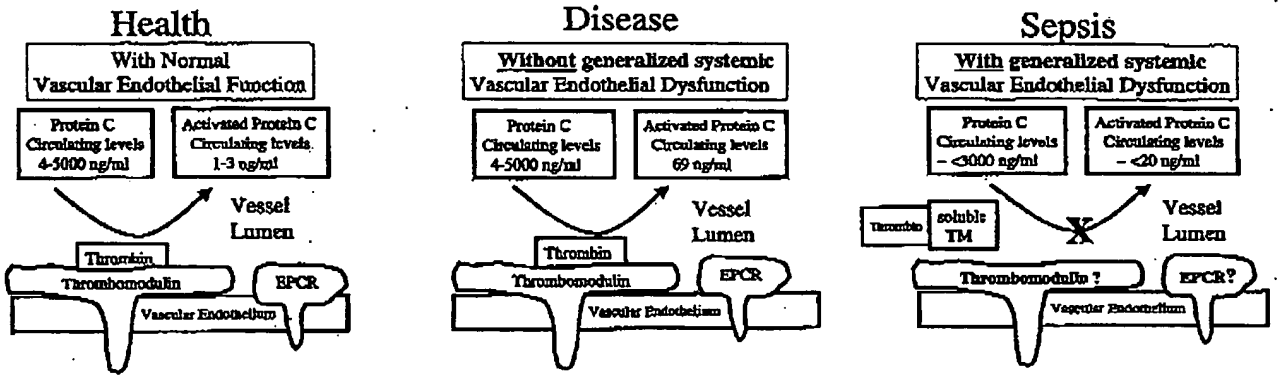
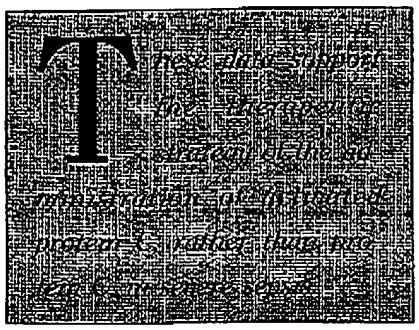


Figure 2. The conversion pathway of protein C to activated protein C by the thrombin-thrombomodulin complex and endothelial protein C receptor (*EPCR*) in health with normal endothelial function (*left*), in disease without generalized systemic endothelial dysfunction (*center*), and in sepsis with generalized systemic endothelial dysfunction (*right*). *X*, lack of or diminished conversion; *TM*, thrombomodulin; *?*, possible alterations in endothelial receptors.



lial dysfunction. In this situation, conversion of protein C to APC is severely limited (<10-fold) due to decreased endothelial surface thrombomodulin, EPCR, or both. Consequently, protein C conversion to APC in sepsis is inadequate, and the homeostatic balance cannot be restored. The intense systemic inflammatory and coagulopathy responses remain unchanged, an imbalance that favors endothelial fibrin deposition, potentially leading to multiple organ failure.

SUMMARY

More than 80% of patients with severe sepsis have decreased levels of endogenous protein C to below the lower limits of normal. Modestly increased (<20 ng/mL) and variable levels of endogenous APC are detectable in <20% of patients with severe sepsis, suggesting that the decreases in endogenous protein C levels cannot be accounted for by increased conversion to endogenous APC. Indeed, the transient elevation of endogenous APC does not parallel the time course of the decrease in protein C. In addition, preliminary clinical data on the decrease in tissue levels of thrombomodulin and EPCR in patients with severe sepsis suggest that the ability to convert protein C to APC may be compromised by sepsis-induced generalized endothelial dysfunction. Only APC, and not protein C, have demonstrated a reduction in mortality in experimental animal models (73–75). Similarly, only APC (drotrecogin alfa [activated]), a recombinant human APC, has been shown in a large phase 3, placebo-controlled, double-blind trial to reduce mortality in patients with severe sepsis (39). No efficacy data from a placebo-controlled, double-blind study are available for protein C in patients with severe sepsis.

Taken together, these data support the therapeutic strategy of the administration of APC, rather than protein C, in severe sepsis. In this way, an active drug is provided to patients without relying on the ability of the patient to convert the inactive protein C to its active form. Additional research is needed in the immediate future to examine the ability of the vasculature and blood components to convert protein C to APC in patients with severe sepsis and to confirm emerging biochemical data.

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